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Hydrolysis of Acid Amides and Amino Acid Amides

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CONTENTS

I. Introduction

This chapter is limited to the enzymes that hydrolyze the primary amides of carboxylic acids and deals mainly with the glutaminases and asparaginase. A number of the peptidases also split the primary amides of amino acids. Table I (pp. 927 and 928, refs. 6-30) shows the enzymes capable of doing this and the substrates studied. More extensive discussion of the peptidases will appear elsewhere in this volume. Discussions of the enzymes that hydrolyze amides have appeared in *Die Methoden der Fermentforschung* (1941, pages 1942–1954)[1] and *Handbuch der Enzymologie* (1940, pages 588–590).[2]

[1] E. Bamann and K. Myrbäck, Die Methoden der Fermentforschung. Thieme, Leipzig, 1941; Academic Press, New York, 1945.

[2] F. F. Nord and R. Weidenhagen, Handbuch der Enzymologie. Leipzig, 1940; Edwards, Ann Arbor, 1943.

No appreciable purification of glutaminase or asparaginase has been accomplished, in part due to the lability of these enzymes and in part due to neglect. The increased amount of study recently devoted to these physiologically important enzymes, which may also be implicated in abnormal metabolism, suggests that procedures for the isolation of purer preparations will soon be available. A number of workers have considered the use of the amidases for analytical purposes[3–5] but so far their use is seriously handicapped by the presence of other enzymes in the preparations employed. A number of investigations have indicated that there are several glutaminases, perhaps as many as three. Further study is required, however, to determine the exact specificity of these enzymes.

II. Methods for Determining Hydrolysis of Amides

1. Analytical Methods

Procedures for following the hydrolysis of amides involve the estimation of the ammonia or the carboxylic acid moiety appearing after hydrolysis. In either case, with more complex substrates like peptides or with crude enzyme preparations there must be assurance that none of the ammonia, or carboxylic acid is formed from substances other than amides. Carboxyl groups can arise from the hydrolysis of peptides and other sources. Am-

[3] W. F. Geddes and A. Hunter, *J. Biol. Chem.* **77,** 197 (1928).
[4] T. L. McMeekin, *ibid.* **123,** lxxxii (1938).
[5] R. M. Archibald, *ibid.* **154,** 657 (1944).
[6] K. Hofmann and M. Bergmann, *ibid.* **130,** 81 (1939).
[7] J. S. Fruton, G. W. Irving, Jr., and M. Bergmann, *ibid.* **141,** 763 (1941).
[8] K. Hofmann and M. Bergmann, *ibid.* **138,** 243 (1941).
[9] M. Bergmann, J. S. Fruton, and H. Pollok, *ibid.* **127,** 643 (1939).
[10] M. Bergmann and J. S. Fruton, *Advances in Enzymol.* **1,** 63 (1941).
[11] G. W. Schwert, H. Neurath, S. Kaufman, and J. E. Snoke, *J. Biol. Chem.* **172,** 221 (1948).
[12] J. S. Fruton and M. Bergmann, *ibid.* **130,** 19 (1939).
[13] J. S. Fruton, G. W. Irving, Jr., and M. Bergmann, *ibid.* **138,** 249 (1941).
[14] G. W. Irving, Jr., J. S. Fruton, and M. Bergmann, *ibid.* **138,** 231 (1941).
[15] G. W. Irving, Jr., J. S. Fruton, and M. Bergmann, *ibid.* **144,** 161 (1942).
[16] J. S. Fruton and M. Bergmann, *ibid.* **145,** 253 (1942).
[17] S. Kaufman, H. Neurath, and G. W. Schwert, *ibid.* **177,** 793 (1949).
[18] R. V. MacAllister, K. M. Harmon, and Carl Niemann, *ibid.* **177,** 767 (1949).
[18a] H. R. Gutmann and J. S. Fruton, *ibid.* **174,** 851 (1948).
[18b] E. L. Smith and M. Bergmann, *ibid.* **153,** 627 (1944).
[19] E. L. Smith and N. B. Slonim, *ibid.* **176,** 835 (1948)
[20] J. S. Fruton, *ibid.* **166,** 721 (1946).
[21] D. Neville-Jones and R. A. Peters, *Biochem. J.* **43,** 303 (1948).
[22] M. Bergmann, L. Zervas, and W. F. Ross, *J. Biol. Chem.* **111,** 245 (1935).
[23] M. Bergmann, L. Zervas, and J. S. Fruton, *ibid.* **111,** 225 (1935).
[24] M. Bergmann and W. F. Ross, *ibid.* **111,** 659 (1935).
[25] M. Bergmann, L. Zervas, and J. S. Fruton, *ibid.* **115,** 593 (1936).
[26] M. Bergmann, J. S. Fruton, and H. Fraenkel-Conrat, *ibid.* **119,** 35 (1937).
[27] J. S. Fruton, *ibid.* **165,** 333 (1946).
[28] J. S. Fruton, *ibid.* **146,** 463 (1942).
[29] A. K. Balls and H. Lineweaver, *ibid.* **130,** 669 (1939).
[30] A. Walti, *J. Am. Chem. Soc.* **60,** 493 (1938).

monia also may have several sources, including peptides.[31] In some cases it might be helpful to determine the decrease in concentration of the substrate.

The appearance of the carboxylic acid component of the substrate can be determined most conveniently by titration. Titration in ethanolic solution by the procedure of Grassman and Heyde[32] is suitable for the microdetermination of the carboxyl groups. This titration, however, does not give satisfactory end points with tyrosine-containing substrates.[13] Ammonia can be determined by any procedure that does not hydrolyze the labile amides. A procedure meeting this requirement and the necessary apparatus were described recently by Archibald.[33] A 5-minute vacuum distillation at 50° and pH 10 is employed, following principles described earlier by Pucher *et al.*,[34] with subsequent determination of the ammonia by nesslerization.[35] Glutamine and urea are not decomposed under these conditions. Determination of ammonia, in the enzymatic hydrolysis of glutamine by direct nesslerization after deproteinization is described[36] for use when the concentration of interfering substances (urea, creatinine, amino acids) is small and when a distillation apparatus is not available. Gorr and Wagner found[37] that formamide and oxamide were decomposed by the conditions which they employed (potassium carbonate, 40°) for ammonia determination in enzymatic hydrolyzates. With these amides, direct nesslerization was used. The increased decomposition of glutamine by heat, with the release of ammonia in the presence of salts, particularly phosphate,[38] is pertinent to the determination of ammonia by methods employing elevated temperatures. McIlwain,[39] in his studies of the bacterial decomposition of glutamine, and Krebs,[40] in his studies of glutamine formation and hydrolysis by animal tissues, employed the apparatus of Parnas and Heller[41] for distillation of the ammonia with subsequent estimation by titration or nesslerization.

Methods for the determination of the substrate glutamine have been reviewed recently.[42, 38] A gasometric procedure[38] is based on the carbon dioxide liberated with ninhydrin before and after the transformation of the glutamine to pyrrolidonecarboxylic acid and ammonia by heating at pH 6.5. Other methods of interest utilize the enzyme glutaminase,[5, 36] which

[31] J. M. Gonçalves, V. E. Price, and J. P. Greenstein, *J. Natl. Cancer Inst.* **8**, 31 (1948).
[32] W. Grassman and W. Heyde, *Z. physiol. Chem.* **183**, 32 (1929).
[33] R. M. Archibald, *J. Biol. Chem.* **151**, 141 (1943).
[34] G. W. Pucher, H. B. Vickery, and C. S. Leavenworth, *Ind. Eng. Chem., Anal. Ed.* **7**, 152 (1935).
[35] F. C. Koch and T. L. McMeekin, *J. Am. Chem. Soc.* **46**, 2066 (1924).
[36] R. M. Archibald, *J. Biol. Chem.* **154**, 643 (1944).
[37] G. Gorr and J. Wagner, *Biochem. Z.* **266**, 96 (1933).
[38] P. B. Hamilton, *J. Biol. Chem.* **158**, 375 (1945).
[39] H. McIlwain, *Biochem. J.* **40**, 460 (1946).
[40] H. A. Krebs, *ibid.* **29**, 1951 (1935).
[41] J. K. Parnas and J. Heller, *Biochem. Z.* **152**, 1 (1924).
[42] R. M. Archibald, *Chem. Revs.* **37**, 161 (1945).

will be discussed later, and a microbiological technique[43] for the determination of glutamine. For the latter procedure a strain of β-hemolytic streptococci is employed, and methionine sulfoxide (0.01 *M*) is added to prevent the synthesis of glutamine from glutamic acid. As little as 5 millimicromoles of glutamine can be determined.

Vickery and associates,[44] in a paper comparing the relative stability of glutamine and asparagine at various pH values, describe the determination of both glutamine and asparagine by preferential hydrolysis at pH 6.5 and in 1 *N* sulfuric acid, respectively. No specific method for asparagine is available.

Bergell and von Wülfing[45] describe changes in optical rotation on the enzymatic hydrolysis of L-leucinamide. Study of this phenomenon for other amides might provide a means of following enzymatic hydrolysis quantitatively.

2. Assay of Enzyme Activity

An assay method for asparaginase is described by Grassmann and Mayr.[46] With their procedure, a unit of asparaginase is defined as the amount of enzyme that will hydrolyze half of 0.005 mole of asparagine in 10 ml. in 2 hours at pH 8 and 40°. The hydrolysis was determined by titration in ethanol with 0.05 *N* potassium hydroxide.

Archibald[5] describes the assay of glutaminase, which is given here in detail.

Into each of seven test tubes measure, in the following order, 0.5 ml. 1 *M* phosphate (pH 7.2), 1.0 ml. 0.0025 *M* glutamine solution, and 0.5 ml. of the glutaminase solution. All solutions are brought to 38° before they are added, and the tubes are kept at 38°. At intervals of 2, 5, 10, 15, 20, and 30 minutes, the action of the enzyme in the respective tubes is stopped by addition of 1 ml. 0.1% bromosulfalein solution. A blank is included in which the glutamine solution is replaced by water. The blank is incubated for 15 minutes. As soon as the enzyme action in each tube is stopped, the tube is stoppered and immersed in ice water, and kept there until the ammonia is distilled. The chilling retards both the spontaneous evolution of ammonia from the glutamine left unchanged by the enzyme and the catalytic breakdown of amide groups of protein by the bromosulfalein. The contents of each tube are washed with 2 ml. water into a micro ammonia apparatus,[33] and the ammonia is distilled, nesslerized, and measured against standards containing 0 to 0.07 mg. ammonia nitrogen.[36] The results are plotted on coordinate paper, milligrams ammonia nitrogen as ordinate and minutes as abscissa. By interpolation, the number of minutes required for the formation of 0.01 mg. ammonia nitrogen is determined. A unit of glutaminase is defined as the amount of enzyme that will liberate this amount of ammonia nitrogen

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[43] J. A. Roper and H. McIlwain, *Biochem. J.* **42**, 485 (1948).

[44] H. B. Vickery, G. W. Pucher, H. E. Clark, A. C. Chibnall, and R. G. Westall, *ibid.* **29**, 2710 (1935).

[45] P. Bergell and H. von Wülfing, *Z. physiol. Chem.* **64**, 348 (1910).

[46] W. Grassmann and O. Mayr, *ibid.* **214**, 185 (1933).

in 10 minutes. In the example shown in Fig. 1, 1.43 units of glutaminase are present in the 0.5-ml. sample employed. In view of the linearity of hydrolysis with time, a single incubation period, up to 15 minutes, could be employed, with only slightly less accurate results.

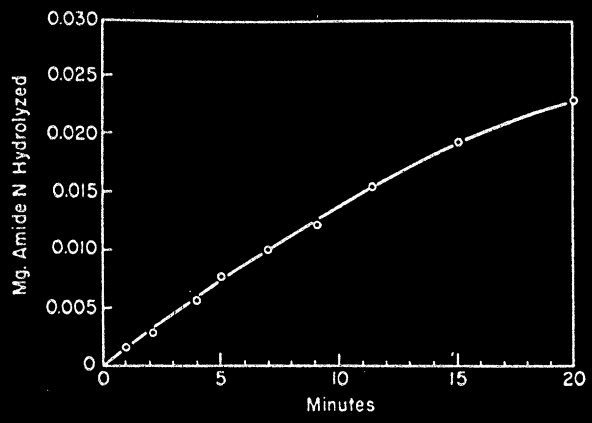

FIG. 1. Ammonia formation in assay of glutaminase.[6]

III. Peptidases Capable of Hydrolyzing Amides

The peptidases capable of hydrolyzing amides are listed in Table I. In a few cases closely related substrates not hydrolyzed are listed to bring out the specificity of the action. In general, the types of compounds hydrolyzed indicate the specificity of the particular enzyme. Trypsin, for example, hydrolyzes peptides in which the amino acid contributing the carboxyl at the point of hydrolysis is arginine or lysine. Furthermore, the amino group of this amino acid must be in a peptide bond. The information tabulated in Table I was obtained almost entirely by Bergmann and associates in their elucidation of the action of proteolytic enzymes, with simple peptides as substrates. Several recent papers summarize their studies.[10, 47, 48]

The trypsinases are akin to trypsin only with respect to the substrates hydrolyzed, since in contrast to trypsin they act only in the presence of an activator like cysteine, and at an optimal pH of about 5. The papains and the cathepsins are most active at this pH also.

Leucine aminopeptidase is widespread, occurring in animal and plant tissues and in bacteria. It is distinguished by its activation with manganese or magnesium salts. In most studies of this enzyme, the substrates employed have been leucylglycine and leucyldiglycine. A summary of studies of this enzyme has appeared recently.[49]

Hydrolysis in most instances is strictly limited to the compounds con-

[47] J. S. Fruton, *Cold Spring Harbor Symposia Quant. Biol.* **6**, 50 (1938).
[48] M. Bergmann, *Advances in Enzymol.* **2**, 49 (1942).
[49] M. J. Johnson and J. Berger, *ibid.* **2**, 69 (1942).

TABLE I

ACTION OF PEPTIDASES ON VARIOUS AMINO ACID AMIDES WITH RELEASE OF AMMONIA

Enzyme	Source	Substrate	Action	Ref.
Trypsin, crystalline	Beef pancreas	Benzoyl-L-lysinamide	Hydrolysis	6,7
Trypsin, crystalline	" "	Benzoylglycyl-L-lysinamide	"	6,8
Trypsin, crystalline	" "	Benzoylglycyl-(ε-carbobenzoxy)-L-lysinamide	No hydrolysis	6
Trypsin, crystalline	" "	Benzoyl-L-argininamide	Hydrolysis	7–9
Trypsin, crystalline	" "	Benzoylglycyl-L-argininamide	"	8
Trypsin, crystalline	" "	Benzoyl-L-histidinamide	No hydrolysis	10
Trypsin, crystalline	" "	α-*p*-Toluenesulfonylargininamide	Hydrolysis	11
Trypsinases	Beef spleen	Benzoyl-L-argininamide	"	7,12–14
"	Swine kidney	Benzoyl-L-argininamide	"	7,15
"	Beef spleen	Benzoyl-L-lysinamide	"	7
Chymotrypsin, crystalline	Beef pancreas	Glycyl-L-tyrosinamide	"	16,17
Chymotrypsin, crystalline	" "	Glycyl-L-phenylalaninamide	"	16
Chymotrypsin, crystalline	" "	L-Tyrosinamide	"	16
Chymotrypsin, crystalline	" "	L-Phenylalaninamide	"	16
Chymotrypsin, crystalline	" "	L-Tyrosylglycinamide	"	16,18
Chymotrypsin, crystalline	" "	Glycylglycinamide	"	16
Chymotrypsin-like	Swine kidney			18*a*
Leucine aminopeptidases	Swine intestinal mucosa	L-leucinamide	"	18*b*,19
Leucine aminopeptidases	Swine intestinal mucosa	Glycyl-L-leucinamide	"	19
Leucine aminopeptidases	Swine intestinal mucosa	L-Glutamyl-L-leucinamide	"	19
Leucine aminopeptidases	Rabbit and human skin, rabbit lung	L-Leucinamide	"	20

TABLE I—(*Continued*)

Enzyme	Source	Substrate	Action	Ref.
Leucine aminopeptidases	Rat skin	"	"	21
Leucine aminopeptidases	Beef spleen	"	"	7,10,12,13
Leucine aminopeptidases	" "	D-Leucinamide	No hydrolysis	10
Leucine aminopeptidases	Beef and swine kidney	L-Leucinamide	Hydrolysis	7
Papain	Papaya	α-Benzoyllysinamide	Hydrolysis	22
"	"	ε-Carbobenzoxylysinamide	No hydrolysis	22
"	"	α-Benzoylglycyl-ε-carbobenzyoxy-lysinamide	Hydrolysis	22
"	"	Benzoylglycinamide	"	14,23–25
"	"	Benzoyl-L-isoglutamine	"	14,23
"	"	Carbobenzoxyisoglutamine	"	25,26
"	"	Carbobenzoxy-L-glutamic acid diamide	"	27
"	"	Benzoyl-L-arginin-amide	"	9,14,15
"	"	L-leucinamide	"	10
"	"	Benzoyl-L-leucin-amide	"	25
"	"	Benzoyl-D-leucin-amide	No hydrolysis	25
"	"	Carbobenzoxy-L-serin-amide	Hydrolysis	28
Papain, crystalline	"	Benzoylglycinamide	"	29
Ficin, crystalline	Fig	Benzoyl-L-arginin-amide	"	14
Ficin, crystalline	"	Benzoylglycinamide	"	30
Bromelin	Pineapple	Benzoylglycinamide	No hydrolysis	26
"	"	Benzoyl-L-arginin-amide	Hydrolysis	9
Cathepsins	Beef spleen	Benzoylglycinamide	"	12
"	Rabbit serum	Benzoylglycinamide	"	20
"	Beef spleen	Carbobenzoxy-L-isoglutamine	"	12
"	" "	Carbobenzoxy-L-serinamide	"	28

taining the L-amino acids, as shown in Table I for leucinamide and benzoyl-leucinamide. An intestinal mucosa preparation[185] showed slight activity with D-leucylglycine, but this was lost on purification of the leucine peptidase, suggesting that the activity was due to a distinct enzyme. An extract of Bashford mouse carcinoma hydrolyzed D- and L-leucinamide at the same rate.[50]

Additional details regarding these enzymes appear elsewhere in this treatise.

IV. Asparaginase

1. Distribution and Specificity

Asparaginase is widely distributed. It occurs in fungi (*Penicillium camemberti*[51] and *Aspergillus niger*[52, 53]), tissues of the horse and pig,[54] the rabbit,[40] the rat,[55–59] and the mouse,[60] in birds and in serum of the guinea pig,[61] in fish livers,[62] but apparently not in the livers of frogs kept in captivity,[62] in plants (barley rootlets),[63] bakery and brewery yeasts,[3, 46, 64, 65] *Torula utilis*[4, 37, 64] and various strains of bacteria.[66, 67] Gorr and Wagner[37] found that the production of asparaginase by *Torula* is determined by the source of nitrogen in the medium, α-amino acids having the greatest effect (see also discussion of acetamidase, Sect. VI). They also reported the curious result that *Torula* grown in ammonium sulfate and then suspended in 10% glucose for 24 hours contains an active asparaginase. They attributed this to the release of stimulatory nitrogenous compounds. The influence of the nitrogen in the medium was noted with bacteria[67] and yeast [42] also, organic nitrogen being the best source. However, *Aspergillus niger* produced asparaginase with only inorganic nitrogen.[52]

Price and Greenstein[59] reported that rat liver and kidney actively hydrolyzed asparagine; rat spleen, pancreas, and muscle acted to a less degree

[50] J. S. Fruton, G. W. Irving, Jr., and M. Bergmann, *J. Biol. Chem.* **132**, 465 (1940).
[51] A. W. Dox, *ibid.* **6**, 461 (1909).
[52] K. Schmalfuss and K. Mothes, *Biochem. Z.* **221**, 134 (1930).
[53] D. Bach, *Compt. rend.* **187**, 955 (1928).
[54] O. von Fürth and M. Friedmann, *Biochem. Z.* **26**, 435 (1910).
[55] J. P. Greenstein and C. E. Carter, *J. Natl. Cancer Inst.* **7**, 57 (1946).
[56] C. E. Carter and J. P. Greenstein, *ibid.* **7**, 433 (1947).
[57] M. Errera and J. P. Greenstein, *ibid.* **7**, 437 (1947).
[58] J. M. Gonçalves, V. E. Price, and J. P. Greenstein, *ibid.* **7**, 281 (1947).
[59] V. E. Price and J. P. Greenstein, *ibid.* **7**, 275 (1947).
[60] M. Errera and J. P. Greenstein, *ibid.* **7**, 285 (1947).
[61] A. Clementi, *Arch. intern. physiol.* **19**, 369 (1922).
[62] G. Steensholt, *Acta Physiol. Scand.* **8**, 342 (1944).
[63] C. E. Grover and A. C. Chibnall, *Biochem. J.* **21**, 857 (1927).
[64] G. Gorr and J. Wagner, *Biochem. Z.* **254**, 1 (1932).
[65] D. Hiwatashi, *Tôhoku J. Exptl. Med.* **42**, 1 (1942); *Chem. Abstracts* **42**, 5067 (1948).
[66] S. Utzino and M. Imaizumi, *Z. physiol. Chem.* **253**, 51 (1938).
[67] G. Busch, *Biochem. Z.* **312**, 308 (1948).

and brain not at all. The kidney of the rabbit had slight activity and liver none under the conditions employed. The activity of mouse liver was much greater than that of rat liver.[60]

Grassmann and Mayr[46] made the first extensive study of the specificity of an extremely active preparation of yeast asparaginase. The following compounds were studied because of their relation to the asparagine (A) molecule: A-glycine, A-dialanine, chloracetyl-A, glycyl-A, mono- and diamides of succinic acid, α-A, β-aminobutyric acid amide, hydroxy-A derivatives, and aspartic acid diamide. None was hydrolyzed except the last, which was hydrolyzed one-tenth as fast as A. The authors concluded from the hydrolysis of the diamide and the resistance of A-glycine and A-dialanine that the effect of substitution of the carboxyl group was merely quantitative. In view of the frequent occurrence of α-amino acid amidases (see Sect. III and VI), however, the significance of the hydrolysis of the diamide can be decided only when the pure enzyme is available. These preparations hydrolyzed glutamine to some degree, but this hydrolysis was inhibited by pyrophosphate, whereas asparagine hydrolysis remained. Krebs[40] first showed that the asparaginase and glutaminase activities of rabbit tissues represented distinct enzymes on the basis of distribution, the ratio of the respective activites was 1:100 in kidney and 4:1 in liver extracts.

2. Preparation and Purification

The yeast asparaginase preparation of Grassmann and Mayr[46] appears to be the most active that has been described. The amount of enzyme in the yeast seemed to be related to the strain and the time of year. Probably nutrition was also a factor, in view of its influence on *Torula utilis*.[37] Grassmann and Mayr, however, considered that the method chosen for preparing the enzyme was of most importance, the principal factor being the avoidance of a low pH. This has been observed for the bacterial asparaginase also.[67] A procedure used by Grassmann and Mayr[1, 46] involves treating the yeast ("*Weissbier hefe*") with toluene, washing with water, and maintaining a pH of 8.5 for 48 hours. After centrifugation, 0.2 ml. supernatant solution, from a volume of 400 ml. containing 400 g. (wet weight) of fresh yeast, hydrolyzed 80% of 0.0001 mole of asparagine in 2.0 ml. in 2 hours at 40°.

Asparaginase has been extracted from barley rootlets with water and concentrated by precipitation with ethanol.[63] Ethanol, as well as acetone, was deleterious to the yeast enzyme[3]; extraction of fresh yeast with water was unsuccessful, but water or glycerol was effective after the yeast was dried. Grassmann and Mayr[46] report that much more enzyme could be extracted by their procedure than by glycerol. The enzyme could be extracted more effectively from fish livers with 10% sucrose than with 80% glycerol.[62] The

culture medium of wild yeast proved to be a good source of asparaginase after the culture had stood for 3 days.[4]

Geddes and Hunter[3] found that the enzyme from yeast could be precipitated with safranine and that the precipitate contained 72% of the activity, with only 15% of the solids in the original extract. Grassmann and Mayr[46] found that asparaginase was not adsorbed on kaolin or alumina C_γ at neutrality. It was adsorbed on kaolin at pH 5, however, and to a greater extent than polypeptidase, but there was not a clean separation of the two enzymes, and this low pH is deleterious to asparaginase.

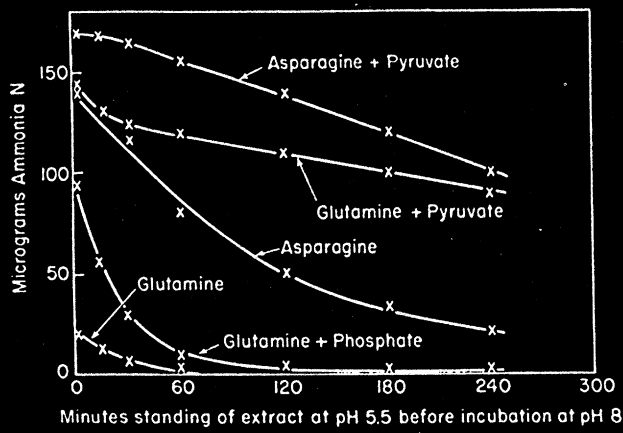

FIG. 2. Hydrolysis of asparagine and glutamine by rat liver extracts[57] with and without added phosphate and pyruvate, after standing at pH 5.5.

3. Stability and Effect of Various Reagents

Grassmann and Mayr[46] found the yeast asparaginase extremely unstable; at 0° and pH 7 to 8, 30% of the activity was lost in 24 hours. At 0° and pH 5, more than 70% of the activity was lost in 30 minutes. The enzyme appeared to be more stable in the presence of the substrate. This has also been noted by Bach.[53] Errera and Greenstein[57] found that rat liver extracts still had some activity after 4 hours at 25° at pH 5.5 (see Fig. 2).

Yeast asparaginase is completely inhibited[46] by 0.0001 *M* Ag^+ and Hg^{++}; hydrogen cyanide (0.04 *M*), hydrogen sulfide (0.004 *M*), and pyrophosphate (0.02 *M*) are not inhibitory to asparaginase, whereas they inhibit yeast di- and polypeptidase and can be used to enhance the specificity of the enzyme preparation. Archibald, however, reports[5] that potassium cyanide is inhibitory to kidney asparaginase. Ammonium aspartate is inhibitory to the wild yeast enzyme[68] in concentrations exceeding 0.015 *M*, but aspartic acid

[68] T. L. McMeekin, *J. Biol. Chem.* **123**, lxxxii (1938).

does not inhibit the rat liver enzyme.[40] Phosphate, arsenate, and sulfate (all 0.02 *N*) have no effect on the hydrolysis by rat liver and brain extracts.[69] The activity of dialyzed asparaginase preparations from wild yeast[68] in phosphate buffers increases with ionic strength up to a value of 0.3. Some of the properties of liver and brain asparaginase from various species have been reported.[70]

4. Kinetics of Hydrolysis

The optimum pH of asparaginase from a variety of sources[3, 52, 57, 67] is about 8. The single exception was the enzyme from barley,[63] which showed an optimum at 7, but in this case the hydrolysis was continued over a period of 12 days. Grassmann and Mayr[46] did not determine the optimum pH, but pH stability studies led them to use pH 8 in their assays.

There is a strong affinity of the enzyme for the substrate[52]; there is no decrease in the activity when the substrate concentration is lowered from 0.1 to 0.025 *M*[46, 67] The hydrolysis is proportional to time and concentration of enzyme[3, 46, 52, 68] and increases with temperature in the expected fashion (25 to 35°),[68] (20 to 40°),[46] and has an optimum temperature[52] of 33°.

V. Glutaminase

1. Distribution and Specificity

The ability to hydrolyze glutamine is possessed by animal tissues,[40, 59, 69] higher plants,[42, 63] wild yeast,[68] and some bacteria.[71] *Proteus morganii* contains an especially active enzyme, hydrolyzing in about an hour its own weight of glutamine.[71] 60% of this enzyme, which is more active at pH 5 than at 7.6, is obtained in soluble form. Rapid glutaminase action has also been observed in strains of *Clostridium welchii*[72, 73] and its analytical use described.[73] Streptococci deamidate glutamine only when there is an accompanying glycolysis,[74] and extracts containing glutaminase cannot be prepared from them.[71] It was concluded that *Staphylococcus aureus* did not contain glutaminase, since glutamine inhibited the growth of this organism.[75] However, the observed inhibition is probably due to impurities in the glutamine used.[76] The growth of several strains of staphylococci was not affected by glutamine.[71] Yeast extracts have been reported by some work-

[69] J. P. Greenstein and F. M. Leuthardt, *Arch. Biochem.* **17**, 105 (1948)
[70] D. Hiwatashi, *Tôhoku J. Exptl. Med.* **41**, 298, 384 (1941); *Chem. Abstracts* **42**, 8231 (1948).
[71] H. McIlwain, *J. Gen. Microbiol.* **2**, 186 (1948).
[72] H. A. Krebs, *Biochem. J.* **42**, v (1948).
[73] H. A. Krebs, *ibid.* **43**, 51 (1948).
[74] H. McIlwain, *ibid.* **40**, 67 (1946).
[75] N. Grossowicz, *J. Biol. Chem.* **173**, 729 (1948).
[76] D. E. Hughes, *ibid.* **176**, 1473 (1948).

ers[3, 46] to hydrolyze glutamine, but others[5] could find no activity. McIlwain concludes[71] that there is a real but slight activity. In view of the effect of the nitrogen component of the medium on the production of asparaginase by *Torula utilis*, yeasts, and bacteria (Sect. IV), this factor should be considered here also.

Krebs found[40] that extracts of tissues from the rabbit, guinea pig, rat, cat, ox, and sheep hydrolyze glutamine. Liver, kidney, retina, brain, spleen, lung, and muscle were studied. Greenstein and associates[59, 60] have studied various tissues from the rat, rabbit, and mouse. Summarizing, it has been

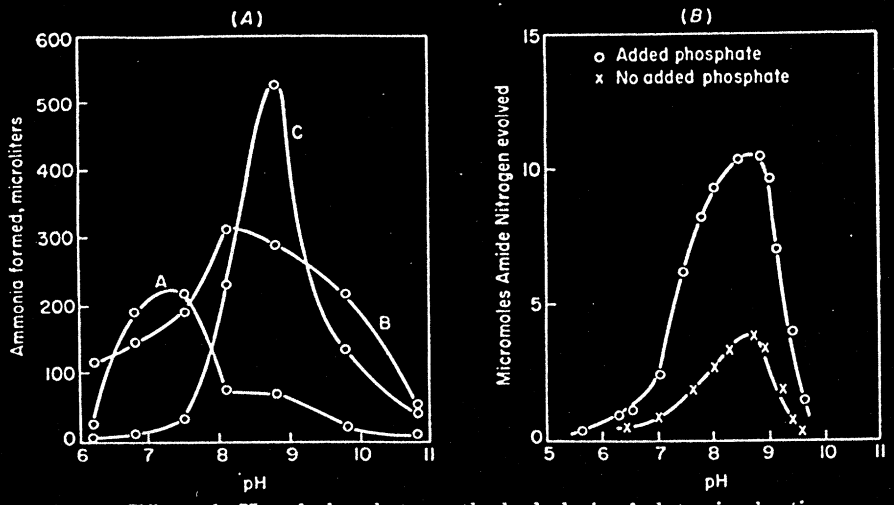

FIG. 3. Effect of pH and phosphate on the hydrolysis of glutamine by tissue extracts. (*A*) Hydrolysis by guinea pig liver (A), sheep kidney (B) and guinea pig brain (C) extracts.[40] (*B*) Hydrolysis by rat brain extracts.[69]

found that in most species extracts from kidney are more active than those from liver. Muscle extracts are consistently low in activity. Archibald found[5] that dog kidney is a much better source of glutaminase than beef kidney; the enzyme is absent from dog plasma. Glutaminase is present in human kidney,[42] but the concentration is one-tenth to one-hundredth of that found in dog kidney.

Tomato, zuccini, and perennial rye grass contain glutaminase, localized in the roots.[42] The concentration of the enzyme is increased greatly if the plants are grown in soil treated with ammonium glutamate.

Krebs' studies[40] indicated that there are two different glutaminases; one, predominating in liver, has an optimal pH of 7 to 8 (see Fig. 3), and is not inhibited by glutamic acid; the other, predominating in brain and retina, has an optimal pH of 8 to 9, and is strongly inhibited by both isomers of glutamic acid; 0.046 *M* glutamic acid, for example, was completely

inhibitory to the enzyme in pig retina. Archibald[5] from results of studies on pH optima also concluded that there are two glutaminases. Greenstein and associates,[69] on the basis of the effect of phosphate, have come to the same conclusion. The first type of enzyme was not affected, but the second type with the higher pH optimum was stimulated as much as fivefold by phosphate (0.02 *M*) (see Fig. 3).

In investigating the specificity of glutaminase, Krebs[40] found that phenacetyl- and benzolyglutamine were not hydrolyzed by kidney or brain extracts nor were the peptides glutaminylglycine and glutaminylglutamic acid, results suggesting that both α-amino and carboxyl groups must be free for hydrolysis of the amide. Isoglutamine was only slowly hydrolyzed by brain extracts. Carter and Greenstein,[56] however, found that rat liver extracts hydrolyzed isoglutamine more rapidly than glutamine and that they hydrolyzed chloroacetylglutamine to some degree. Archibald[5] investigated the action of his enzyme preparation on a large number of nitrogenous compounds principally to determine whether other ammonia-forming enzymes were present. The slow hydrolysis of citrulline ($HOOCCHNH_2$-$(CH_2)_3NHCONH_2$) was considered of interest in view of similarities to glutamine in structure. In view of the multiplicity of "glutaminases," it appears that further studies on specificity will be most profitable when the enzymes are available in purer form than the tissue extracts so far employed.

2. Preparation and Purification

Glutaminase is currently prepared by suspending the tissues in water, grinding with sand, and centrifuging to remove the coarser particles. Archibald,[5] who was interested in a preparation for analytical purposes, employed 0.04 *M* potassium cyanide adjusted to pH 7.2 as the suspending medium (3 parts to 1 of tissue), since it enhanced the activity of some preparations (up to sevenfold) and depressed asparaginase and deamination activities. 0.5 ml. of such a preparation completely hydrolyzed[36] 3.6×10^{-6} *M* glutamine in a volume of 2 ml. in 30 minutes at pH 7.2 and 38°. A commercial enzyme preparation, Polidase S, presumably made from mold, contained considerable glutaminase. Also present were proteinase, phosphatase, and adenosine deaminase, limiting its usefulness for glutamine analyses.[36]

Archibald reported[5] the only attempts to purify tissue glutaminase; isoelectric precipitation, acetone at 0°, and precipitation with safranine (see Sect. IV-2) were unsuccessful. The purification and some of the properties of glutaminase from *Clostridium welchii* have been briefly described.[77]

[77] D. E. Hughes and D. H. Williamson, *Biochem. J.* **43**, xlv (1948).

3. Stability and Effect of Various Reagents

Errera and Greenstein[57] reported the deleterious effect of acid (pH 5.5) on rat liver glutaminase (see Fig. 2). The preparations of Archibald[5] retained adequate activity for only 24 hours at 0°.

The effects of cyanide, glutamic acid, and phosphate on glutaminase have already been mentioned. The effects of phosphate (0.01 *M*) on hydrolysis by rat brain extracts is shown in Fig. 3*B*, and by rat liver extracts in Fig. 2. Phosphate increases the hydrolysis of glutamine much more than pyruvate when fresh tissue extracts are employed,[69] (personal communication) although it is not evident from the data in Fig. 2.[57] Arsenate and sulfate also stimulate the hydrolysis of glutamine by tissue extracts; nitrate, chloride, and bicarbonate have no effect.[56, 69] Ethyl phosphonate is slightly stimulatory; methyl arsonate and benzene sulfonate have no effect.[69] Hamilton[38] observed that phosphate enhanced the transformation of glutamine by heat to pyrrolidonecarboxylic acid (PCA) and ammonia. This was eliminated as the enzymatic mechanism, since PCA was not formed from glutamine nor was PCA hydrolyzed to glutamic acid.[56] Woodward and Reinhart also found[78] that PCA was not transformed into glutamic acid by tissue extracts but that, in the enzymatic hydrolysis of glutathione, PCA was formed. The formation was strongly dependent on pH, being maximal at high pH values. Glutamic acid was the product formed at low pH values.

Archibald[5] reported inhibition of glutaminase by the following compounds (potassium cyanide and phosphate were present): *p*-benzoquinone (0.00014 *M*, 73%), bromosulfalein (0.002 *M*, 100%), and atabrine (0.0093 *M*, 100%). The purified glutaminase from *Clostridium welchii* is activated by chloride ions.[77] The action of this enzyme is accelerated by cetyltrimethylammonium bromide[73, 77, 79] apparently by increasing the affinity of the enzyme for the substrate.[79]

4. Kinetics of Hydrolysis

The effect of pH has been noted (Fig. 3). Studies on the effect of substrate concentration on the hydrolysis of glutamine have not been extensive. Archibald[36] has shown that *dog* kidney extracts rapidly effect complete hydrolysis of glutamine, hence there must be a strong affinity between this enzyme and the substrate even at low concentrations of the latter. Krebs[40] on the contrary found that with *pig* kidney extracts hydrolysis had almost stopped at less than 50% hydrolysis of 1.5×10^{-4} mole of glutamine in 10 ml., because of accumulation of glutamic acid. In part this difference may

[78] G. E. Woodward and F. E. Reinhart, *J. Biol. Chem.* **145**, 471 (1942).
[79] D. E. Hughes, *Biochem. J.* **43**, xvi (1948).

be due to the difference in the concentrations of the substrate (0.0018 *M* *vs.* 0.015 *M*, respectively), but the distribution of the two types of glutaminases in various organs may differ with the species. In an experiment with guinea pig brain extract and concentrations of glutamine of 0.005, 0.01, and 0.02 *M*, Krebs[40] found that the release of ammonia in 30 minutes was in the ratio of 1 to 3.4 to 9.2. Apparently in this instance the affinity between enzyme and substrate is slight.

Krebs[40] found that initial velocities of hydrolysis are approximately proportional to the enzyme concentration. Archibald[5] found that for 10 minutes the extent of hydrolysis is proportional to time (see Fig. 1).

VI. Other Amides Hydrolyzed by Enzymes

The hydrolysis of amides of carboxylic acids and amino acid amides other than those mentioned above will be discussed in this section. The hydrolysis of leucinamide, benzoylargininamide, and other complex amino acid amides has been briefly referred to in Section III, since the enzymes that hydrolyze these amides are primarily peptidases. However, when the enzymes have not been studied in pure form, as is the case with papain and many of the enzymes in animal tissues, the limits of specificity are difficult to determine. Because of this lack of knowledge of the precise limits of peptidases and amidases, some of the substrates mentioned in Section III will be discussed here also. The hydrolysis of aliphatic and cyclic amides can be regarded as effected by acylases also. Additional information on this type of enzyme is given elsewhere in this volume.

Information about the hydrolysis of amides of carboxylic acids is scarce, since these compounds were in most instances used only incidentally to determine the specificity of amino acid amides like asparagine and glutamine. In most cases no hydrolysis was observed. Recently Bray and associates[80] have begun studies in this neglected field. They have correlated their *in vitro* results with the *in vivo* disposition of various amides, particularly the benzamides.

1. Distribution and Specificity of the Enzymes

a. Hydrolysis of Aliphatic Amides

The aliphatic amides that have been tested with various enzyme preparations are listed in Table II. With two exceptions, the results are negative; a rabbit liver extract hydrolyzed propionamide[80]; a *Torula utilis* preparation hydrolyzed some of the amides, but did not hydrolyze others.[61] McMeekin[4] reported that aliphatic amides were not hydrolyzed by extracts of a wild strain of *Torula*. The hydrolysis effected by *Torula*[64] was somewhat greater

[80] H. G. Bray, S. P. James, B. E. Ryman, and W. V. Thorpe, *ibid.* **42**, 274 (1948).
[81] M. Errera and J. P. Greenstein, *J. Natl. Cancer Inst.* **8**, 71 (1948).

with preparations dried in vacuum at 25° than with the fresh material, presumably due to increased permeability of the cell membrane (with asparagine the increase in hydrolysis was threefold). Acetone drying, however, was deleterious.[37] The *Torula* activity was present in autolyzates and passed through a Chamberland filter candle.[37]

In a significant paper, Gorr and Wagner[37] demonstrated the importance of the nitrogen source for the production of asparaginase and acetamidase by *Torula utilis*. The basic medium contained 2% glucose, 0.06% KH_2PO_4 and 0.03% magnesium sulfate, the nitrogen compound supplying 0.05% nitrogen. When ammonium sulfate was the source of nitrogen, acetamidase

TABLE II

EFFECT OF VARIOUS ENZYME PREPARATIONS ON ALIPHATIC AMIDES[a]

Substance	Source of Enzyme Preparation							
	Rabbit liver[80]	Mouse and rat tissues[81]	Barley rootlets[63]	Yeast[3]	Yeast[16]	Yeast[64]	*Torula utilis*[37, 64]	*Aspergillus niger*[52]
Formamide	−			−		−	−	
Acetamide	−	−	−	−	−	−	+	−
Acetomethylamide							−	
Chloroacetamide		−						
Propionamide	+		−	−	−	−	+	
α-Chloropropionamide		−						
Valeramide				−				
Succinamide				−	−	−	−	
Lactamide						−	+	
Oxamide				−		−	−	−

[a] No hydrolysis is indicated by −; measurable hydrolysis by +. Where there is no − or +, the substances were not tested.

was absent, and asparaginase was neglible; the α-amino compounds glycine, alanine, and aspartic acid were good producers of asparaginase, whereas acetamidase production was best with the amides urea and acetamide. By the use of autolyzates and boiled extracts, cell permeability and inhibitors were eliminated as the cause of these results.

Further study of the phenomena observed by Gorr and Wagner[37] appears to have promise for the preparation of amidases for analytical purposes. Such a study has been initiated,[4] and the inclusion of asparagine itself, together with one other amino acid, in the medium resulted in excellent production of asparaginase by a strain of *Torula*.

In general, amidases for aliphatic amides appear not to occur naturally in plant and animal tissues and yeasts. *Torula utilis*, with a suitable source of nitrogen, can produce such enzymes.

b. Hydrolysis of Cyclic Amides

Rabbit liver extracts hydrolyzed benzamide definitely and *p*-nitrobenzamide almost completely under the conditions employed.[80] Other benzamide derivatives (nitro-, hydroxy-, and amino-; *o*-, *m*-, or *p*-) were hydrolyzed very little. These experiments were in general agreement with *in vivo* experiments, in which the compound was fed and the urine analyzed. The single exception was phenylacetamide, which was not hydrolyzed *in vitro*, but *in vivo* appeared in the urine as phenylaceturic acid.[82] (For other *in vivo* studies see Bray *et al.*[83–85])

Benzamide is also hydrolyzed by extracts of horse kidney,[86] and it is slowly hydrolyzed by preparations of *Penicillium camemberti*.[51] Salicylamide is not hydrolyzed by yeast extract.[3] Benzamide and phenylacetamide, however, are hydrolyzed.[65] Pyrrolidonecarboxamide is hydrolyzed by a *Vibrio* enzyme preparation but not by animal tissues.[87]

Acetanilide is not hydrolyzed by *Torula utilis* extract.[37] It is readily hydrolyzed by ground rat liver and kidney, and the enzyme is also reported to be present in the livers of the dog, cat, rabbit, and ox.[88] *In vivo* studies,[89] however, suggest that the enzyme is not present in rabbit or man but that it is present in rat, dog, and pigeon. The *in vivo* hydrolysis of toluidineacetic acid compounds in the rabbit is negligible.[90]

c. Hydrolysis of Amino Acid Amides

Waldschmidt-Leitz and associates[91] reported the hydrolysis of glycinamide and leucinamide by swine intestinal preparations (erepsin); yeast preparations hydrolyzed the second but not the first compound. A number of amides of amines (decarboxylated amino acids, for example, glycyldecarboxyalanine, $NH_2CH_2CONHC_2H_5$) were hydrolyzed by the intestinal but not the yeast preparations. Grassmann and Dyckerhoff[92] reported that yeast polypeptidase hydrolyzed glycylleucinamide, glycyldecarboxyleucine, glycinamide, and leucinamide; these compounds were not hydro-

[82] H. G. Bray, F. C. Neale, and W. V. Thorpe, *Biochem. J.* **40**, 134 (1946).
[83] H. G. Bray, B. E. Ryman, and W. V. Thorpe, *ibid.* **41**, 212 (1947).
[84] H. G. Bray, H. J. Lake, F. C. Neale, W. V. Thorpe, and P. B. Wood, *ibid.* **42**, 434 (1948).
[85] H. G. Bray, B. E. Ryman, and W. V. Thorpe, *ibid.* **43**, 561 (1948).
[86] H. Waelsch and A. Busztin, *Z. physiol. Chem.* **249**, 135 (1937).
[87] U. Senji, D. Hiwatashi, and O. Kimio, *Tôhoku J. Exptl. Med.* **42**, 46 (1942); *Chem. Abstracts* **42**, 5067 (1948).
[88] H. O. Michel, F. Bernheim, and M. L. C. Bernheim, *J. Pharmacol. Exptl. Therap.* **61**, 321 (1937).
[89] J. N. Smith and R. T. Williams, *Biochem. J.* **42**, 538 (1948).
[90] H. G. Bray and W. V. Thorpe, *ibid.* **43**, 211 (1948).
[91] E. Waldschmidt-Leitz, W. Grassmann, and A. Schäffner, *Ber.* **60B**, 359 (1927).
[92] W. Grassmann and H. Dyckerhoff, *ibid.* **61B**, 656 (1928).

lyzed by dipeptidase or protease preparations. Glycylleucinamide is split at both the peptide and the amide bonds. The hydrolysis of the leucine compounds is due to the leucinepeptidase (see Sec. III). Glycinamide is slowly hydrolyzed by human intestinal juice.[93]

Errera and Greenstein[81] found that the following amides were hydrolyzed (only the L-isomer) by rat and mouse tissues: glycine, DL-alanine, DL-valine, DL-α-amino-*n*-butyric acid, DL-leucine, and DL-norleucine. Glycinamide was hydrolyzed most slowly, and the others more rapidly in the order listed. Kidney and liver were the most active tissues; brain and spleen were intermediate; muscle and pancreas were the least active. Carter and Greenstein[56] found that isoglutamine was rapidly hydrolyzed by rat liver extracts. Fruton and Bergmann[12] (see Sec. III) reported that carbobenzoxy-L-isoglutamine was hydrolyzed by beef spleen at pH 5; the hydrolysis was accelerated by cysteine. The free isoglutamine was not employed. Papain also hydrolyzed acylated isoglutamine[14, 23, 25, 26] (see Sec. III), and it is stated that two —C=O·NH— bonds were essential for hydrolysis of the amides. Fruton[27] prepared L-glutamine by hydrolysis of carbobenzoxy-L-glutamic acid diamide with papain. Greenstein and Leuthardt[69] reported on the hydrolysis of L-isoglutamine and the amides of glycine, DL-alanine, DL-leucine, and L-benzoylarginine by rat liver and brain extracts.

Bergmann and associates[10, 94] found that papain, bromelin, and pig liver contain an enzyme that hydrolyzes anilides and phenylhydrazides of the amino acids. Because of the low solubility of these compounds, they can be synthesized from their components by enzymatic catalysis, benzoylglycine anilide, for example, being formed from benzoylglycine and aniline. Only the L-isomers of the amino acids participate in this reaction, and it has been made the basis of a procedure for resolving DL-leucine, with papain as the catalyst.[94] The method can be used to resolve DL-methionine also.[95]

At present it is not known whether the enzymes that attack amino acid amides are other than peptidases. Errera and Greenstein[81] have found that an α-amino group is essential for the hydrolysis of the amides mentioned earlier. If there is a more restricted specificity, the question remains whether there are one or several α-amidases. In part the data of Errera and Greenstein favored a single enzyme (hydrolysis rates for different amides with different tissues were in the same order), but comparison of pairs of amides (alanine and leucine amides with liver and kidney) showed that this conclusion was not fully justified. Fractionation studies and eventual purification will be necessary to answer this question.

[93] P. A. Levene, H. S. Simms, and M. H. Pfaltz, *J. Biol. Chem.* **70**, 253 (1926).
[94] M. Bergmann and H. Fraenkel-Conrat, *ibid.* **119**, 707 (1937).
[95] C. A. Dekker and J. S. Fruton, *ibid.* **173**, 471 (1948).

2. Properties of the Enzymes

The enzyme from rabbit liver which hydrolyzed benzamide, described by Bray and associates,[80] had a pH optimum at 7.4; the activity remained unchanged after a 17-hour dialysis. Hydrolysis of the amides studied stopped at about 10 hours, however, even though incomplete, suggesting that the enzyme had lost its activity or that the products of the hydrolysis were inhibitory. The latter may be a contributing factor, since phenylacetic acid was inhibitory to the hydrolysis of benzamide and *p*-nitrobenzamide.

pH-activity data were obtained by Errera and Greenstein[81] for the hydrolysis of glycine, alanine, and leucine amides. The optimum in every case was about pH 9; it was not changed by the addition of manganese (0.002 *M* final concentration), which increased the rate of hydrolysis. The optimum pH and the effect of manganese are to be expected[96] (Sec. III) for the hydrolysis of leucinamide by leucine peptidase, but whereas this enzyme is reported to have a high affinity for the substrate,[96] Errera and Greenstein found that for the above substrates the activity with respect to concentration was still increasing at high concentrations of the substrates (0.091, 0.246, and 0.077 *M*, respectively).

The hydrolysis of the above-mentioned amides[81] by dialyzed rat liver extracts was not increased by cysteine or ascorbic acid at pH 5.2 or 7.0. Fruton *et al.*[13] have reported such an effect with beef spleen extracts at pH 5.2. Errera and Greenstein[81] also reported data for the hydrolysis of these amides by neoplastic tissue extracts.

Greenstein and Leuthardt[69] in studying the enzymatic hydrolysis of various amino acid amides were interested in the effects of phosphate, arsenate, sulfate, and pyruvate on the reaction. The hydrolysis by rat liver and brain extracts of the L-isoglutamine, and the amides of glycine, DL-alanine, and DL-leucine was not affected by these salts. The hydrolysis of L-benzoylargininamide was inhibited by phosphate and arsenate. At pH 6.2, the optimum for this substrate, and at a substrate concentration of 0.025 *M*, inhibition was almost complete with 0.25 M phosphate. The pH optimum was not shifted by the phosphate. The optimum pH for the hydrolysis of isoglutamine is reported to be about 8.[69]

VII. Enzymatic Hydrolysis of Amides in the Presence of Pyruvate (Dehydropeptidase)

In addition to the specific hydrolysis of asparagine (Sect. IV) and glutamine (Sec. V), a less specific hydrolysis takes place in the presence of liver and kidney extracts and pyruvate.[55, 58, 59, 97, 98] This hydrolysis is believed to involve an interreaction of the amide and pyruvic acid to give

[96] M. J. Johnson, G. H. Johnson, and W. H. Peterson, *ibid.* **116**, 515 (1936).
[97] J. P. Greenstein and C. E. Carter, *ibid.* **165**, 741 (1946).
[98] J. M. Gonçalves and J. P. Greenstein, *J. Natl. Cancer Inst.* **7**, 269 (1947).

a dehydropeptide, which is subsequently hydrolyzed by a dehydropeptidase to give aspartic or glutamic acid, ammonia, and pyruvic acid:

$$RCONH_2 + R'COCOOH \rightarrow RCON{=}CR'COOH$$

$$RCON{=}CR'COOH \xrightarrow{\text{dehydropeptidase}} RCO{\cdot}OH + R'COCOOH + NH_3$$

The effect of the pyruvate is maximal at a ratio of 2 moles to 1 mole of amide.[55] The optimal pH for the pyruvate effect[98] in rat liver is 7.2, the same as for dehydropeptidase activity, whereas the optimal pH for activity of glutaminase and asparaginase is about 8. Pyruvate also increases the deamidation of chloroacetylglutamine in both liver and kidney extracts.[59] Pyruvate has no effect on the deamidation of isoglutamine, chloroacetylasparagine, or glycylasparagine in either tissue. Extracts of spleen, brain, muscle, and pancreas show no significant pyruvate effect with either glutamine or asparagine.[59] The stimulation of deamidation by pyruvate is also observed when pyruvate is furnished by a concommitant reaction such as the enzymatic hydrolysis of glycyldehydroalanine and other dehydropeptides.[58, 98] The dehydropeptidase (pyruvate effect) is much more resistant to acid (pH 5.5) than is asparaginase or glutaminase[57] (Fig. 2). The dehydropeptidase literature has been reviewed recently.[99]

VIII. Physiological Importance of the Amidases

The widespread occurrence of asparagine in plants led Grover and Chibnall[63] to a study of the deamidase in barley rootlets. This was the first demonstration of the presence of asparaginase in higher plants. No special physiological role for the amidase, however, has yet been found. Archibald[42] in a recent review compares the probable roles of glutamine and asparagine in animals and plants, respectively. An altered concentration of asparaginase has been observed in neoplastic tissues.[60] Asparaginase in pathological conditions has been studied.[70]

Only recently a specific physiological involvement of glutaminase has been demonstrated by Van Slyke and associates.[100] The amide nitrogen of glutamine was found to be the main source of urinary ammonia, and kidney glutaminase presumably is involved in this transfer. Kidneys of premature infants, who are prone to acidosis, contain little glutaminase.[42]

Ten of eighteen kidneys showing high glutaminase activity were from patients with neoplastic growths in organs other than the kidney.[42] Changes in the concentration of glutaminase have been found[60] in neoplastic hepatic tissues in the rat and mouse relative to fetal, adult, and regenerating hepatic tissues. In a number of neoplastic tissues, the predominant glutaminase

[99] J. P. Greenstein, *Advances in Enzymol.* **8**, 117 (1948).

[100] D. D. Van Slyke, R. A. Phillips, P. B. Hamilton, R. M. Archibald, P. H. Futcher, and A. Hiller, *J. Biol. Chem.* **150**, 481 (1943).

apparently is the type stimulated by phosphate.[101] In human kidneys with involvement such as chronic hemorrhagic nephritis or arteriosclerotic alterations, glutaminase could not be found.[42] Archibald[42] in a thorough review discusses the physiological roles of the glutamine–glutaminase system.

Addendum

Since this article was prepared a number of papers have appeared concerning amidases. Studies of the action of trypsin[102, 103] and chymotrypsin[104, 105, 106, 107] on substrates listed in Table I have been reported. Chymotrypsin hydrolyzes nicotinyl-L-tyrosylhydrazide.[108] Of great interest is the discovery[102, 105] that these enzymes hydrolyze esters as well as amides. The leucine aminopeptidase in mouse brain hydrolyzes the ethyl ester of L-leucine, as well as the amide.[109] The specificity[110] and inhibition[111] of this enzyme have also been studied. Papers have appeared on the hydrolysis of benzoylarginine amide by extracts of hog kidney,[111] of salivary glands of *Drosophila melanogaster*,[112, 113] of the liver and kidney of the rabbit,[114] and normal and tuberculous tissues of rabbits.[114a] Aqueous extracts of swine kidney cortex activated with manganese rapidly hydrolyze L-methionineamide.[115] Carbobenzoxy-L-methioninamide is hydrolyzed rapidly by both papain and ficin; the D-isomer is not hydrolyzed.[115] Carbobenzoxy-L-threoninamide is hydrolyzed slowly by papain and ficin.[115] The action of ultraviolet light on ficin is reported; benzoylargininamide was used as the substrate.[116] *S*-Benzylhomocysteine has been resolved into its optical isomers by the formation of anilides; papain was the catalyst.[117] Formation of anilides of alanine, leucine, glycine, valine, and phenylalanine by catalysis with papain has been studied in detail.[118, 119] The phenylhydrazides

[101] J. P. Greenstein and F. M. Leuthardt, *J. Natl. Cancer Inst.* **8**, 161 (1948).
[102] G. W. Schwert and M. A. Eisenberg, *J. Biol. Chem.* **179**, 665 (1949).
[103] K. M. Harmon and C. Niemann, *ibid.* **178**, 743 (1949).
[104] S. Kaufman and H. Neurath, *ibid.* **180**, 181 (1949).
[105] G. W. Schwert and S. Kaufman, *ibid.* **180**, 517 (1949).
[106] S. Kaufman and H. Neurath, *Arch. Biochem.* **21**, 245 (1949).
[107] S. Kaufman and H. Neurath, *ibid.* **21**, 437 (1949).
[108] R. V. MacAllister and C. Niemann, *J. Am. Chem. Soc.* **71**, 3854 (1949).
[109] I. Krimsky and E. Racker, *J. Biol. Chem.* **179**, 903 (1949).
[110] E. L. Smith and W. J. Polglase, *ibid.* **180**, 1209 (1949).
[111] O. Schales and B. R. Hill, *Arch. Biochem.* **22**, 366 (1949).
[112] E. K. Patterson, M. E. Dackerman, and J. Schultz, *J. Gen. Physiol.* **32**, 607 (1949).
[113] E. K. Patterson, M. E. Dackerman, and J. Schultz, *ibid.* 623 (1949).
[114] J. Schultz, *J. Biol. Chem.* **178**, 451 (1949).
[114a] C. Weiss and J. Schultz, *Proc. Soc. Exptl. Biol. Med.* **72**, 236 (1949).
[115] C. A. Dekker, S. P. Taylor, Jr., and J. S. Fruton, *J. Biol. Chem.* **180**, 155 (1949).
[116] I. Mandl and A. D. McLaren, *Arch. Biochem.* **21**, 408 (1949).
[117] L. J. Reed, A. R. Kidwai, and V. du Vigneaud, *J. Biol. Chem.* **180**, 571 (1949).
[118] S. W. Fox, F. N. Minard, H. Wax, C. W. Pettinga, and J. Strifert, *Federation Proc.* **8**, 198 (1949).
[119] C. W. Pettinga and S. W. Fox, *ibid.* 236 (1949).

of *N*-carbobenzoxy-*o*-fluorophenylalanine can be synthesized by the same technique.[120] In this instance, both L- and D-isomers are formed, although the latter is formed at a slower rate. Synthesis of *N*-carbobenzoxyalanine-phenylhydrazide apparently is limited to the L-isomer.[120]

There now appear to be two asparaginases.[121] One, labile when aqueous extracts of rat liver are heated at 50° for 10 minutes, has been designated asparaginase I. The relatively heat-stable enzyme has been designated as asparaginase II. It is activated by α-ketoisocaproate as well as by pyruvate; the pH value of optimal activity with each compound is 7.5 and 8.0, respectively. The greatest activity is found at a molar ratio of the keto acid to asparagine of about 2.5. Asparaginase II appears to be present only in liver, and not in kidney, spleen, and brain. Sodium lactate has no effect on this enzyme; lactate activates unheated liver extracts about 30% but not extracts from kidney, spleen, and brain.[121] Liver and kidney extracts do not hydrolyze β-L-aspartyl-L-alanine or *N*-carbobenzoxy-L-asparagine[121] A brief reference has been made to the preparation of asparaginase free of glutaminase.[122]

Several reports have appeared on the nonenzymatic desamidation of glutamine.[123, 124] In phosphate buffer at 37°, the hydrolysis is 28% in 20 hours; in veronal buffer the hydrolysis is only 6% in 20 hours.[123] Pyrrolidonecarboxylic acid appears to be the product formed.[123] Studies in greater detail have shown that the phosphate-activated glutaminase I does not catalyze this process, for the mechanisms differ in several ways.[124] The enzymatic reaction yields glutamic acid and ammonia, the nonenzymatic reaction yields pyrrolidonecarboxylic acid and ammonia. The former is accelerated by sulfate but not by borate or bicarbonate, whereas the reverse is true of the nonenzymatic reaction. The enzymatic reaction has a pH optimum of 7.5 to 8.8 in various tissues, with or without the anion present, whereas the nonenzymatic effect of phosphate increases with pH. Chloracetylglutamine does not hydrolyze spontaneously in the presence of phosphate.[124]

Separation of glutaminase I (phosphate activated) and glutaminase II (pyruvate activated) of rat liver has been described.[122] Glutaminase I is obtained in about 30% yield, with a threefold increase in activity, based on the nitrogen content, by centrifuging and washing the insoluble portion of liver homogenates. The soluble glutaminase II is fractionated with ethanol at low temperature; the yield is 20% with a more than tenfold increase in activity. Data are presented that show the effects of pH, time,

[120] E. L. Bennett and C. Niemann, *J. Am. Chem. Soc.* **70,** 2610 (1948).
[121] J. P. Greenstein and V. E. Price, *J. Biol. Chem.* **178,** 695 (1949).
[122] M. Errera, *ibid.* **178,** 483 (1949).
[123] H. G. Bray, S. P. James, I. M. Raffan, and W. V. Thorpe, *Biochem. J.* **44,** 625 (1949).
[124] J. B. Gilbert, V. E. Price, and J. P. Greenstein, *J. Biol. Chem.* **180,** 209 (1949).

concentration of enzyme, and concentration of glutamine on the activity of the purified glutaminase II. The maximal activity of the enzyme reached by increasing the concentration of the glutamine is increased by adding pyruvate. Potassium cyanide and sodium iodoacetate in 0.025 *M* concentration completely inhibit the enzyme, at least in part reversibly. Glutaminase II hydrolyzes isoglutamine, but this hydrolysis is not affected by pyruvate. Several dehydropeptides are hydrolyzed at an appreciable rate. Glutaminase II is activated by α-ketoisocaproate, as well as by pyruvate.[121] The respective pH optima are 7.2 and 8.8, and the molar ratios of keto acid to glutamine are about 3 and 1. Studies of the phosphate activation of glutaminase activity have been extended to species other than the rat.[125] The activity at pH 8.0 in aqueous extracts of rat and mouse kidney, liver, brain, and spleen and of rabbit and guinea pig brain and spleen is greatly increased by added phosphate. Under the same conditions, the activity of extracts of rabbit and guinea pig kidney and liver and of the sedimentable fraction of the same tissues is only slightly increased by added phosphate. The presence of a third glutaminase for which neither phosphate nor pyruvate is required is still a possibility.

Further studies have appeared[126] on the bacterial glutaminases which now have been obtained in 50 to 100% yield in cell-free extracts of *Clostridium welchii*, *Proteus morganii*, and *Escherichia coli*. The activity of the soluble enzyme is greatly increased by cetyl methyl ammonium bromide. This effect becomes smaller as the glutamine concentration is increased, that is, the apparent affinity of the glutaminase for the substrate is increased by adding this detergent. The actual mechanism, however, may be the removal of a competitive inhibitor in the enzyme preparation.

Extensive experiments have been performed to determine whether the enzyme in rabbit liver that hydrolyzes benzamide and *p*-nitrobenzamide, is related to the known amidases.[127] Extracts of rabbit, guinea pig, rat, cat and dog liver and kidney, as well as hog and horse kidney and rabbit brain were tested with the substrates *p*-nitrobenzamide, benzamide, glutamine, asparagine, acetylglycine, acetanilide, hippuric acid, nicotinamide and glycylglycine. Rabbit liver extracts after precipitation with acetone, with acetic acid, and with safranine and after heating at 50° for 30 minutes were tested with many of the same substrates. On the basis of distribution of the enzyme, it was concluded that the benzamidase is probably a distinct enzyme. This enzyme is accelerated by phosphate, as is glutaminase. Acetylglycine and acetanilide appear to be hydrolyzed by different enzymes.

[125] M. Errera and J. P. Greenstein, *ibid.* **178**, 495 (1949).
[126] D. E. Hughes, *Biochem. J.* **45**, 325 (1949).
[127] H. G. Bray, S. P. James, I. M. Raffan, B. E. Ryman, and W. V. Thorpe, *ibid.* **44**, 618 (1949).

Further extensive *in vitro* and *in vivo* studies have appeared[127a] with the aliphatic amides, formamide, acetamide, propionamide, *n*-butyramide and *n*-valeramide. Only the last two are hydrolyzed appreciably by rabbit liver extracts.

A number of papers have appeared on the metabolism of various amides —pyruvamide and acetamide in the rat,[128] amides of nitrobenzoic acids,[129] and amides of toluic acids in the rabbit.[130]

Anterior pituitary growth hormone preparations given to hypophysectomized rats increased the kidney glutaminase.[131, 131a] It has been suggested[131] that the storage of amino nitrogen might be a function of the glutamine-glutaminase system, similar to the role this system plays in the transport and storage of ammonia.[100] Added evidence for this theory is the finding that a cell-free extract of *Proteus vulgaris* catalyzes the exchange of the amide group of glutamine or asparagine with hydroxylamine.[132] A possible role of these amino acid amides in peptide synthesis was visualized.[132] Rat kidney glutaminase does not change in activity during alkalosis (induced by $NaHCO_3$) and acidosis (induced by NH_4Cl).[133] Increased ammonia excretion in this type of acidosis therefore cannot be explained by increase in glutaminase activity. However, a combination of phosphoric and β-hydroxybutyric acids greatly stimulates kidney glutaminase,[134] suggesting a possible relationship to the increased formation of urinary ammonia in diabetic acidosis. The amidases have been studied in cancer formation in rat and mouse liver.[135] Glutaminase I increases greatly, whereas glutaminase II decreases. The final pattern is that of fetal liver. Asparaginase II decreases in cancer formation. This paper[135] summarizes studies on dehydropeptidases and peptidases, as well as amidases, in relation to formation of cancer.

[127a] H. G. Bray, S. P. James, W. V. Thorpe, M. R. Wasdell, and P. B. Wood. *ibid.* 45, 467 (1949).

[128] H. S. Anker and R. Raper, *J. Biol. Chem.* **176**, 1353 (1948).

[129] H. G. Bray, W. V. Thorpe, and P. B. Wood, *Biochem. J.* **44**, 39 (1949).

[130] H. G. Bray, W. V. Thorpe and P. B. Wood, *ibid.* **45**, 45 (1949).

[131] P. D. Bartlett, *Federation Proc.* **8**, 182 (1949).

[131a] P. D. Bartlett and O. H. Gaebler, *J. Biol. Chem.* **181**, 523 (1949).

[132] H. Waelsch, E. Borek, and N. Grossowicz, 54c, Am. Chem. Soc. Proc. Sept. 1949.

[133] P. Handler, F. Bernheim, and M. L. C. Bernheim, *Arch. Biochem.* **21**, 132 (1949).

[134] E. Mylon and J. H. Heller, *Am. J. Physiol.* **154**, 542 (1948).

[135] J. P. Greenstein, P. J. Fodor, and F. M. Leuthardt, *J. Natl. Cancer Inst.* **10**, 271 (1949).